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Asparaginase of *Alcaligenes faecalis*

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Screening was carried out in order to find out bacterial strains that would produce high activity and yield of asparaginase. *Alcaligenes faecalis* was found to have the highest specific and total activity, and chosen for purification and characterization of the enzyme, though almost all the organisms investigated contained measurable quantities of asparaginase. Asparaginase was purified from the cell-free extracts to homogeneity or near homogeneity, and occurrence of two isoenzymes was confirmed, which are designated asparaginase a and asparaginase b. They differ from each other in substrate specificity. Asparaginase a catalyzes predominantly hydrolysis of L-asparagine, and also formation of β-L-aspartylhydroxamate, while D-asparagine, L-glutamine and D-glutamine are hydrolyzed and transferred only slightly. Asparaginase b hydrolyzes L- and D-glutamine as well as L-asparagine, though D-asparagine is rather a poor substrate. These isoenzymes are similar in the other respects, *i. e.*, affinity to the substrates, optimum pH region and molecular weight (a: 63,000, and b: 58,000).

INTRODUCTION

Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1.) catalyzes hydrolysis of L-asparagine to L-aspartate and ammonia. Since the first isolation of asparaginase in 1806 by Vauquelin and Robiquet,[1] the numerous works on the enzyme have been reported as reviewed by Varner,[2] and Sallach and Fahien.[3] The enzyme is widely distributed in animals, plants, and microorganisms. Recently intense interest in asparaginase has been focused on its antilymphoma activity,[4~10] and the large scale preparation of the enzyme of high purity in high yield. The homogeneous or nearly homogeneous enzyme was first prepared from guinea pig serum.[11,12] Microbial asparaginase has been also purified to homogeneity from *Escherichia coli*[13~16] and from *Erwinia carotovora*.[17]

In the present paper, we report distribution of asparaginase in bacteria, its purification from the cell-free extract of *Alcaligenes faecalis*, and some of its properties.

EXPERIMENTAL

Materials

L-Asparagine and L-glutamine were supplied by Kyowa Hakko Kogyo Co., Tokyo. D-Asparagine, D-glutamine, L-theanine and D-theanine, and DEAE-cellulose were products of Ajinomoto Co., Tokyo, and Midori-Juji Co., Osaka, respectively.

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Sephadex G-150 was purchased from Pharmacia, Uppsala, Sweden. γ-Glutamylhydroxamate and β-aspartylhydroxamate were products of Sigma, St. Louis, U. S. A. Hydroxylapatite was prepared according to the method of Tiselius *et al.*[18] The other chemicals were analytical grade reagents.

Microbiological Methods

The bacteria were grown in the medium composed of 1.0% peptone, 0.5% meat extract, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.01% yeast extract, and 0.01% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.2 with sodium hydroxide solution. The cultures were grown at 28° for 18 hours. The cells harvested by centrifugation were washed twice with 0.85% sodium chloride solution.

Analytical Methods

Ammonia was determined calorimetrically with nitroprusside principally according to the procedure of Nagatsu and Yagi,[19] as follows. A 0.5 ml-aliquot of the reaction mixture described below was mixed with 2.0 ml of a fresh mixture of 1 volume of 10% sodium tungstate, and 8 volume of 1/12 N sulfuric acid, followed by centrifugation. To 0.8 ml of the supernatant solution was added succesively 0.2 ml of 1 N sodium hydroxide, 1 ml of phenol reagent composed of 5% phenol and 2% sodium hydroxide, 7 ml of 0.1 M sodium phosphate buffer (pH 9.8), 0.5 ml of 0.05% sodium nitroprusside solution, and 0.5 ml of sodium hypochlorite solution (Cl; about 5%). The sample solution was replaced by water in a blank. After incubation at 37° for 10 min, absorbance was measured at 610 mμ against a blank using a Zeiss PMQ II spectrophotometer.

Hydroxamates were determined according to the method of Lipmann and Tuttle.[20] After the reaction system (1 ml) was mixed with 0.5 ml of 0.6 M trichloroacetic acid to terminate the reaction, 0.5 ml of 0.5 M ferric chloride and 0.5 ml of 2 N hydrochloric acid were added to the reaction mixture. The mixture was centrifuged if necessary. The color intensity developed was measured at 540 mμ. Authentic β-aspartylhydroxamate and γ-glutamylhydroxamate were employed as the standards.

Amino acids were determined spectrophotometrically with ninhydrin after circular paper chromatographical separation by the procedure described previously.[21] Protein was determined by the method of Lowry *et al.*[22] using egg albumin as the standard; with most column fractions, protein solution patterns were estimated by the 280-mμ absorption.

Assay of Asparaginase

Procedure 1. The standard reaction mixture contained 25 μmoles of L-asparagine, 50 μmoles of potassium phosphate buffer, pH 7.4, and enzyme in a final volume of 10 ml. After incubation at 37° for 20 min, 0.5 ml of the reaction mixture was subjected to determination of ammonia as described above.

Procedure 2. The standard reaction mixture consisted of 25 μmoles of L-asparagine (or other amino acid amides), 300 μmoles of hydroxylamine neutralized with sodium hydroxide, and 40 μmoles of potassium phosphate buffer, pH 7.4 in a final volume of 1.0 ml. After incubation was performed at 37° for 20 min, hydroxamate formed was determined as described above.

Procedure 3. After the reaction mixture of procedure 1 was incubated at 37° for 20 min, followed by deproteinization with 50% trichloroacetic acid, an aliquot of solution (10 to 40 μl) was employed for determination of amino acid formed.

One unit of asparaginase is defined as the amount of enzyme that catalyzes the formation of 1.0 μmole of ammonia, hydroxamate or aspartate per min. Specific activity is defined as units per mg of protein.

RESULTS

Distribution of Asparaginase in Various Strains

The activity of asparaginase in various strains of bacteria was investigated by measuring the formation of ammonia from L-asparagine with dialyzed cell-free extracts. As shown in Table 1, the activity was found more or less in the extracts from all the bacterial strains tested except *Proteus mirabilis*. The high specific activity of enzyme occurs in *Proteus vulgaris*, *Alcaligenes faecalis*, *Pseudomonas synxantha*, and *Brevibacterium*

Table 1. Asparaginase Activity of Various Species of Bacteria. The cell-free extracts were employed for assay of asparaginase, which was carried out by procedure 1. Organisms were grown in 100 ml of the medium. The other conditions are described in text.

Strain	Total protein	Specific activity	Total units
Achromobacter liquidum	40.5 mg	0.020	0.80
Achromobacter polymorph	11.3	0.057	0.64
Achromobacter superficialis	47.5	0.077	3.64
Flavobacterium flavescens	60.8	0.068	4.18
Flavobacterium arborscens	6.8	0.091	6.18
Flavobacterium suaveolens	21.7	0.038	0.82
Escherichia coli B-1	48.2	0.080	3.85
Escherichia coli B-3	32.0	0.070	3.63
Aerobacter aerogenes	50.0	0.080	4.00
Erwinia carotovora	24.5	0.080	1.95
Serratia plymuthicum	2.4	0.040	0.96
Serratia marcescens	6.0	0.170	1.04
Proteus mirabilis	31.1	0	0
Proteus vulgaris	34.5	0.350	1.21
Alcaligenes faecalis	33.6	0.600	20.80
Pseudomonas fluorescens	31.5	0.480	15.20
Pseudomonas aeruginosa	46.7	0.060	2.80
Pseudomonas iodium	14.5	0.050	7.25
Pseudomonas segnis	7.8	0.040	3.12
Pseudomonas synxantha	47.3	0.340	16.08
Brevibacterium ammoniagenes	6.1	0.270	1.64
Micrococcus roseus	9.4	0.004	0.04
Sarcina aurantiaca	7.0	0.007	0.05
Protoaminobacter alboflavus	11.4	0.002	0.02

ammoniagenes. The total activity was found high in the extracts from the first three strains. The only slight activity is present in *Micrococcus roseus, Sarcina auranthiaca,* and *Protoaminobacter alboflavus*. In *Pseudomonas aeruginosa* which is known to have the high activity of enzyme catalyzing hydrolysis of glutamine and theanine,[23] the asparaginase activity is not high in both the specific and total activities. *Alcaligenes faecalis* in which asparaginase occurs most abundantly was chosen for the purpose of purification of enzyme.

Purification of Enzyme

All operations, unless otherwise specified, were carried out at 0–5°.

Step 1. The washed cells of *Alcaligenes faecalis* were suspended in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.01% 2-mercaptoethanol and disrupted by treatment for 10 min in a 19-kc Kaijo Denki ultrasonic disintegrator. The intact cells and cell debris were removed by centrifugation at 17,000×g for 30 min.

Step 2. To the supernatant solution was added ammonium sulfate to bring to 20% saturation. The pH was kept between 7.2 and 7.4 with 7% ammonia solution. After the precipitate was removed by centrifugation, the supernatant was brought to 50% saturation with ammonium sulfate, followed by standing the mixture for 30 min. The precipitate obtained was dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.01% 2-mercaptoethanol, dialyzed against the same buffer, and centrifuged to remove the precipitate formed.

Step 3. The supernatant was placed on a column of DEAE-cellulose (10×80 cm) equilibrated with the dialysis buffer. The column was eluted with the same buffer

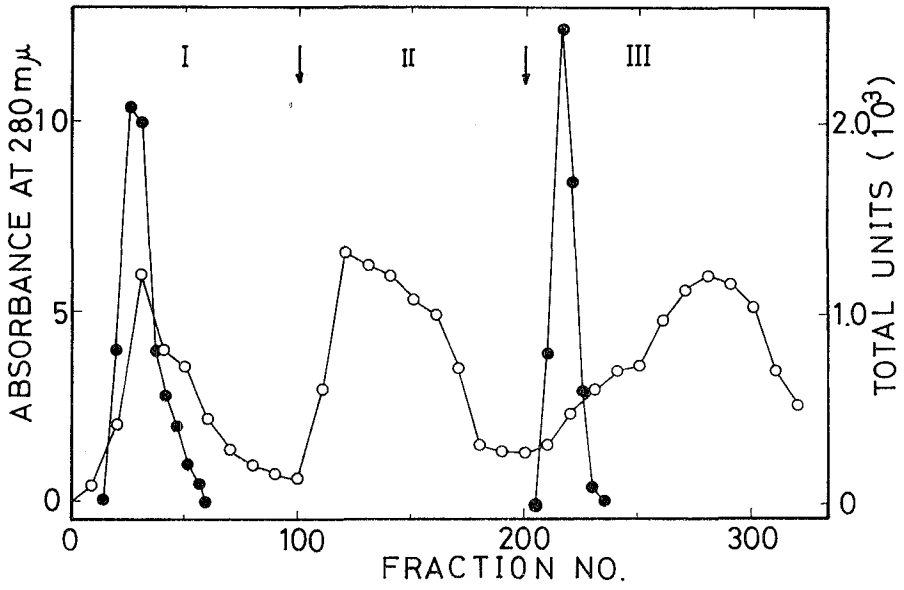

Fig. 1. The elution pattern from a DEAE-cellulose column. The flow rate was approximately 150-ml per hr and 30-ml fractions were collected. (○), Absorbance at 280 mμ; (●), asparaginase activity determined by procedure 1. Other conditions are given in text.

Table 2. Purification of Asparaginase a and b. The enzyme was assayed by procedure 2.

Step	Total protein	Specific activity	Total unit	Yield
Crude extract	193,725 mg	0.47	91,050	100%
20–50% $(NH_4)_2SO_4$	75,328	0.94	70,808	78
DEAE-cellulose column				
Asparaginase a	4,200	13.3	55,963	61
Asparaginase b	620	33.6	20,815	23
Hydroxylapatite column				
Asparaginase a	278	44.0	12,232	13
Asparaginase b	160	23.0	3,680	5
Sephadex G-150 column				
Asparaginase a	140	55.0	7,700	8
Asparaginase b	83	41.0	3,403	4

(I in Fig. 1). The first active fractions (Asparaginase a) was found in the eluate. After the column was washed thoroughly with the buffer containing 0.07 M sodium chloride and 0.01% 2-mercaptoethanol(II), the enzyme was eluted with a linear gradient from 0.01 M potassium phosphate buffer, pH 7.4 containing 0.07 M sodium chloride and 0.01% 2-mercaptoethanol(3 liters) to the same volume of the buffer supplemented with 0.15 M sodium chloride and 0.01% 2-mercaptoethanol (III). These fractions of enzyme are designated asparaginase b. The elution pattern is shown in Fig. 1. The active fractions of asparaginase a and b were pooled independently, concentrated by addition of ammonium sulfate (60% saturation), and dissolved in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01% 2-mercaptoethanol. Both the enzyme solutions were dialyzed thoroughly against the same buffer.

Step 4. The enzyme solutions were applied separately to columns of hydroxylapatite (3×40 cm) equilibrated with the dialysis buffer, and eluted with the same buffer. Two columns were used to purify asparaginase a. All activities of asparaginase a and asparaginase b were found in the eluates. The active fractions of each asparaginase were pooled, concentrated by addition of ammonium sulfate (60% saturation), and dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7.2, containing 0.01% 2-mercaptoethanol.

Step 5. The respective enzyme solutions were placed on Sephadex G-150 columns (2×80 cm) equilibrated with the above-mentioned buffer, and eluted with the same buffer. The active fractions of both asparaginases were combined and concentrated by addition of ammonium sulfate (60% saturation).

The protocol of purification of asparaginase a and b is given in Table 2. Both asparaginase a and b were purified approximately 100-fold with an over-all yield of several per cent.

Purity and Molecular Weight

Disc gel electrophoresis in 7.5% polyacrylamide gel was performed by a modi-

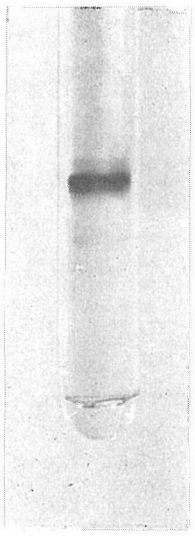

Fig. 2. Disc gel electrophoresis of asparaginase a (24 μg). Electrophoresis was conducted at a current of 2 mA for 2 hr in Tris-glycine buffer (pH 9.0). The direction of migration is from the cathode (top of photo) to the anode. Other conditions are given in text.

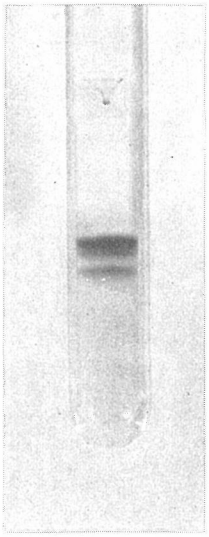

Fig. 3. Disc gel electrophoresis of asparaginase b (26 μg). Conditions are given in Fig. 2.

fication of the procedure of Davis[24] to check the purity of asparaginase a and b obtained as described above. The enzymes were placed on the top of spacer gel in 1 M sucrose. After the run, protein was stained with 1.0% Amido-Schwarz in 7% acetic acid. Destaining was accomplished in 7% acetic acid. The purified asparaginase a is homogeneous by this criterion, while the preparation of asparaginase b obtained contains still a slight amount of impurities, as shown in Figs. 2 and 3.

The molecular weights of asparaginase a and b were determined by chromatography on a Sephadex G-150 column (1.8 × 60 cm) at 8°. A buffer consisting of 0.01 M potassium phosphate, pH 7.4, 0.1 M sodium chloride, and 0.01% 2-mercaptoethanol was used to elute the enzyme. The flow rate was 3 ml per hour and 2.0 ml aliquots of eluate were collected. The Sephadex G-150 column was standardized with catalase, asparaginase of *Escherichia coli*,[16] bovine serum albumin and egg albumin according to the procedure of Determann and Michel.[25] As shown in Fig, 4, the molecular weights of asparaginase a and b were determined to be approximately 63,000 and 58,000 respectively.

Effect of pH on Enzyme Activity

Asparaginase a and b when examined in the presence of acetate, potassium phosphate, carbonate-borate, and pyrophosphate buffers have an optimum reactivity at pH 7.5 and pH 8.2, respectively as shown in Fig. 5.

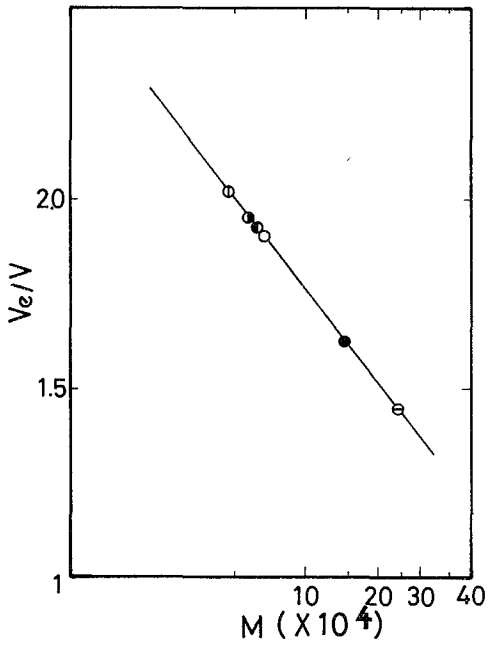

Fig. 4. Molecular weight determination for asparaginase a and b by Sephadex G-150 gel chromatography. The void volume (V) of the column was determined by the elution of a 0.2-ml sample of blue dextran. A 0.5 ml sample containing 5 mg each of the indicated reference proteins was allowed to run into the column and was eluted as shown in text. Samples of asparaginase a and b (0.5 mg each) were eluted from the column in a similar manner. Collected fractions were assayed for both protein and enzyme activity, and a single symmetric peak was obtained. (◐), Egg albumin; (◑), asparaginase b; (◐), asparaginase a; (○), bovine serum albumin; (●), *Escherichia coli* HAP asparaginase; (⊖), catalase.

Effect of Substrate Concentration on Enzyme Activity

The enzyme activities of asparaginase a and b with various concentrations of the substrate were investigated. A plot of reciprocal of the substrate concentration against that of the reaction velocity is given in Fig. 6. The apparent Michaelis constants of asparaginase a and b for L-asparagine were calculated to be 7.15×10^{-3} M and 4.0×10^{-3} M, respectively.

Substrate Specificity

Table II indicates the substrate specificity of asparaginase a and b. Asparaginase a catalyzes overwhelmingly hydrolysis of L-asparagine and formation of β-aspartylhydroxamate, while D-asparagine, L-glutamine and D-glutamine are very poor substrates. On the other hand, asparaginase b hydrolyzes L-glutamine and D-glutamine as well as L-asparagine, the most susceptible substrate, though D-asparagine is only slowly hydrolyzed by the enzyme. The similar results were obtained when asparaginase b was assayed by procedure 2.

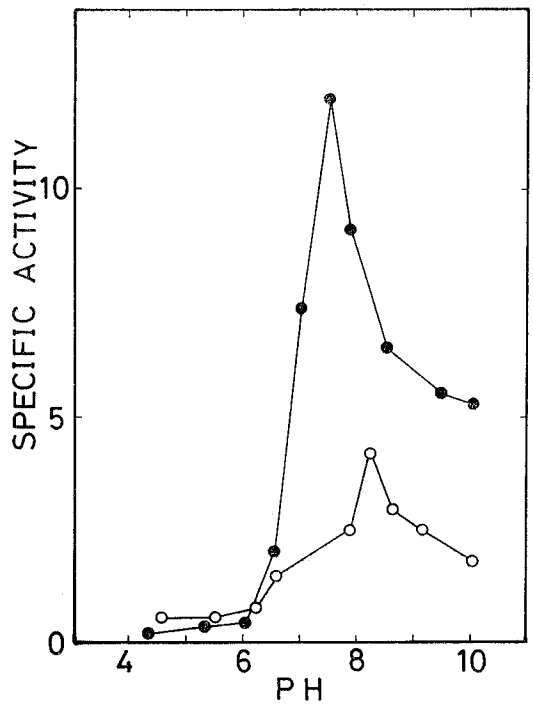

Fig. 5. Effect of pH on activities of asparaginase a (●) and asparaginase b (○). The enzyme activities were determined by procedure 2. The following buffers were employed. Acetate buffer, pH 4.3–5.5; potassium phosphate, pH 5.5–7.8; Na_2CO_3–H_3BO_3–KCl, pH 8.2–9.6; pyrophosphate, pH 9.5–10.5.

Table 3. Substrate Specificity of Asparaginase a and b.
Enzyme activity was determined by procedure 2.

Substrate	Relative activity	
	Asparaginase a	Asparaginase b
L-Asparagine	100	100
D-Asparagine	1.5	29
L-Glutamine	8.2	91
D-Glutamine	8.2	88

DISCUSSION

Recently Peterson and Ciegler[26] reported on L-asparaginase production by various bacteria strain; screening was carried out in an effort to find organisms that would produce high yields of L-asparaginase. Although almost all of the bacterial species screened contained measurable quantities of L-asparaginase, a few strains of *Erwinia aroideae* and one of *Hydrogenomonas eutropha* were reported to produce considerab-

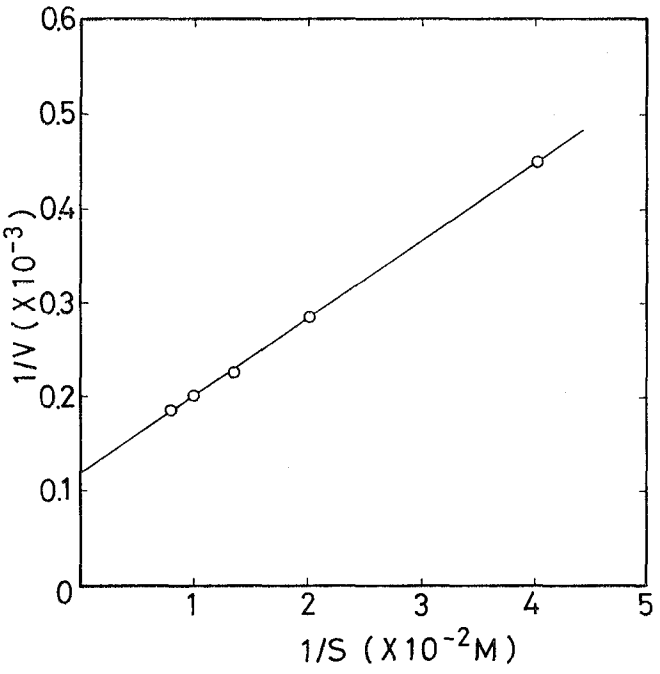

Fig. 6. Determination of the Michaelis constant of asparaginase a for L-asparagine. Assay was performed by procedures 2 and 3. Other conditions are given in text.

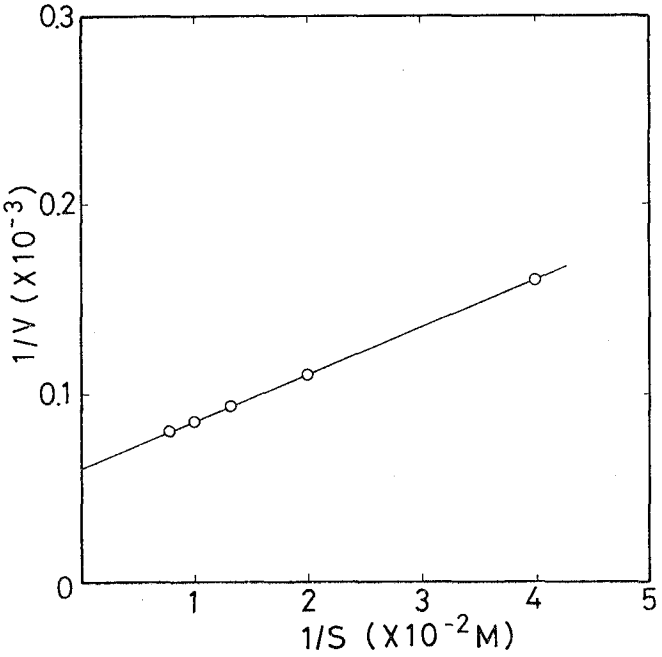

Fig. 7. Determination of the Michaelis constant of asparaginase b for L-asparagine. Assay was performed by procedures 2 and 3. Other conditions are given in text.

ly large amounts under their test system. In the present studies also substantially all the strains investigated were shown to have the activity of asparaginase. Such widespread occurrence of asparaginase suggests ubiquitous importance of the enzyme in bacterial metabolism. *Alcaligenes faecalis* which is the highest in specific and total activities among the strains has been selected for purification and characterization of the enzyme.

In *Alcaligenes faecalis* occurrence of two isoenzymes of asparaginase was found. One of them, asparaginase a, is not adsorbed by DEAE-cellulose under the conditions employed, and the other, asparaginase b, is adsorbed and eluted with the buffer supplemented with higher concentration of sodium chloride. Both of them were highly purified; asparaginase a is homogeneous by criterion of disc gel electrophoresis, and asparaginase b is nearly homogeneous. *E. coli* was shown by several workers[7,8,10,27] to produce two distinct asparaginase (I and II) which differ in a number of properties, perhaps most significantly in their markedly different affinities for asparagine. Asparaginase II inhibits more effectively the growth of transplantable mouse and rat tumors *in vivo*, it is also active against lymphoma in the dog[29] and against human lymphoblastic leukemia.[9] Nikolaev, Evseev, Tyupanova and Abdumalikov[28] reported that two isoenzymes of asparaginase (A and AG) in *Pseudomonas sp.* were demonstrated by chromatography on columns of Sephadex G-100 and DEAE-cellulose. Asparaginase A catalyzes exclusively hydrolysis of L-asparagine, while asparaginase AG is capable of hydrolyzing D-asparagine and L-glutamine as well as L-asparagine. Asparaginase a and b described here may correspond to asparaginase A and AG with regard to substrate specificity, though asparaginase a of *Alcaligenes faecalis* catalyzes also hydrolysis of D-asparagine, and L- and D-glutamine at very low rate. Both the isoenzymes are closely similar in their affinity for the substrates, and optimum pH regions.

The molecular weight of asparaginase a and b are approximately 63,000 and 58,000, respectively, when determined by column chromatography on a Sephadex G-150. These values are considerably small compared to those of asparaginase from other organisms investigated so far, *e.g.*, 141,000 in *E. coli* HAP,[16] 130,000 in *Erwinia carotovora*,[30] 255,000 in *E. coli* B, which is decreased to 132,000 by dilution or by addition of sodium chloride,[14,31] and 133,000 in also *E. coli* B.[32] Asparaginase from *E. coli* was reported to be dissociated to subunits whose molecular weight is approximately 65,000.[31] The 65,000-dalton subunit is composed of at least two smaller subunits. Asparaginase a and b from *Alcaligenes faecalis* are unique in this regard, though possibility that these isoenzymes may be artifacts resulted from dissociation during purification is not excluded.

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